

Isolation of X and Y Chromosome-Specific DNA Markers From a Liverwort, *Marchantia polymorpha*, by Representational Difference Analysis

Masaki Fujisawa, Kiwako Hayashi, Tomohisa Nishio, Tomoyuki Bando, Sachiko Okada, Katsuyuki T. Yamato, Hideya Fukuzawa and Kanji Ohyama

Laboratory of Plant Molecular Biology, Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan

Manuscript received May 24, 2001
Accepted for publication August 21, 2001

ABSTRACT

The liverwort *Marchantia polymorpha* has X and Y chromosomes in the respective female and male haploids. Here we report the successful exploitation of representational difference analyses to isolate DNA markers for the sex chromosomes. Two female-specific and six male-specific DNA fragments were genetically confirmed to originate from the X and Y chromosomes, respectively.

IN a few dioecious plants, morphologically distinct sex chromosomes (AINSWORTH *et al.* 1998; JUAREZ and BANKS 1998), which are expected to play a crucial role in sex determination and differentiation, have been observed. Thus far, several repeated sequences and a few genes have been isolated from sex chromosomes of two plant species: *Silene latifolia* (DONNISON *et al.* 1996; BUŽEK *et al.* 1997; SCUTT *et al.* 1997; GUTTMAN and CHARLESWORTH 1998; DELICHÈRE *et al.* 1999; MATSUNAGA *et al.* 1999) and *Rumex acetosa* (SHIBATA *et al.* 1999). However, the molecular function of sex chromosomes in sex determination and differentiation in plants still remains largely unknown.

The liverwort *Marchantia polymorpha* is dioecious and haploid during most of its life cycle. *M. polymorpha* has an X chromosome in female and a Y chromosome in male individuals, in addition to the common eight autosomes (BISCHLER 1986). This X/Y exclusiveness can be exploited to isolate sex chromosome-specific DNA fragments for both of the sex chromosomes. In contrast, in most dioecious plants, DNA fragments unique to an X chromosome cannot be readily identified because the X chromosome is present in both males and females.

We previously isolated a P1-derived artificial chromosome (PAC) clone specific to the Y chromosome, pMM4G7, from the male genomic library of *M. polymorpha* (OKADA *et al.* 2000). Furthermore, we found that the sequences cloned in pMM4G7 have accumulated in a distinct region of the Y chromosome (OKADA *et al.* 2001). The X chromosome, on the other hand, carries 17S rDNA sequences (NAKAYAMA *et al.* 2001). The sequence composition of the other regions of the Y and X chromosomes, respectively, has remained unclear.

To obtain DNA markers unique to these sex chromosomes in *M. polymorpha*, we performed representational difference analysis (RDA) using male and female total DNAs digested with *Bam*HI, *Hind*III, or *Bgl*II.

MATERIALS AND METHODS

Plant materials: Female and male thalli of *M. polymorpha* (E lines; OKADA *et al.* 2000) were used for RDA and as parents for linkage analysis. F₁ progenies were generated by crossing male and female individuals as follows: sperm cells were collected by adding sterilized water onto a male sex organ and then recovering the water, which was poured onto a female sex organ 5 mm in height. The resulting spores were cultivated in the conditions described by OKADA *et al.* (2000).

Representational difference analysis: The RDA procedure was essentially as described by LISITSYN *et al.* (1993). Briefly, amplicons were generated from 1 µg of male and female total DNA, respectively. Adaptors were removed from tester and driver by ultrafiltration (SUPREC-02, Takara Biomedicals, Kusatsu, Japan) after digestion with the respective restriction enzymes. Tester was ligated to the J-adaptor for the first and third RDA rounds, and the N-adaptor for the second and fourth rounds. A mixture of tester and driver in 8 µl of 30 mM N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid) and 3 mM EDTA (pH 8.0) (STRAUS and AUSUBEL 1990) overlaid with 35 µl of mineral oil (Sigma-Aldrich, Steinheim, Germany) was denatured at 98° for 5 min; 2 µl of 5 M NaCl was then added, and the DNA was allowed to hybridize at 67° for 36 hr. Forty micrograms of the driver DNA was used for each round, while the tester DNA was added in the following amounts: when RDA was performed with *Bam*HI or *Bgl*II, 400 ng tester was added for the first round, 50 ng for the second, and 100 pg for the third; with *Hind*III, 400 ng tester was added for the first round, 100 ng for the second, 400 pg for the third, and 5 pg for the fourth. To the subtractions with amplicons from the female DNA as driver and with amplicons from the male DNA as tester, 1 µg DNA from pMM4G7 was added to the driver preparation to eliminate those Y chromosome-specific repetitive sequences (OKADA *et al.* 2000, 2001) from the male tester DNA.

PCR assay: Twenty nanograms of *M. polymorpha* total DNA was amplified in a 20-µl reaction containing 0.5 units of AmpliTaqGold (Applied Biosystems, Foster City, CA), 2 mM of MgCl₂,

Corresponding author: Kanji Ohyama, Laboratory of Plant Molecular Biology, Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan.
E-mail: kohyama@lif.kyoto-u.ac.jp

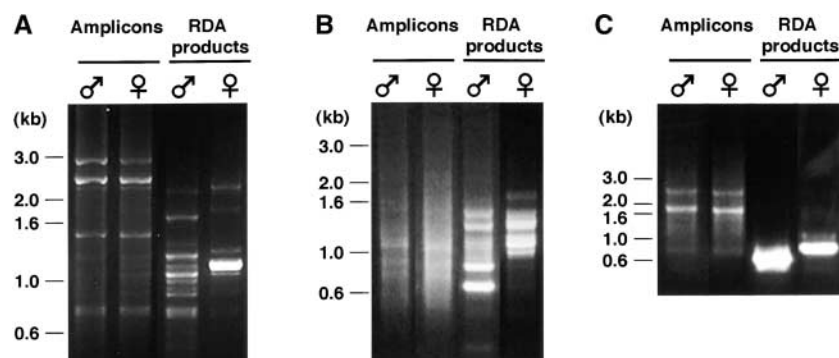


FIGURE 1.—RDA products from *M. polymorpha* male and female genomic DNA digested with *Bam*HI (A), *Hind*III (B), and *Bgl*II (C), respectively. The third-round RDA products (A and C) and the fourth-round RDA products (B) were electrophoresed.

0.5 μ M of each primer, and 0.2 mM of each dNTP. PCR cycles were programmed as follows: 10 min at 96° followed by 35 cycles (96° for 1 min, 50–54° for 1 min, according to the melting temperature of respective primers sets, and 72° for 1 min) with a GeneAmp PCR System 9700 (Applied Biosystems). The primers, which can detect a sequence present in both male and female (CDPK-Ex1; NISHIYAMA *et al.* 1999), were used as a positive confirmation of male and female PCR amplifications (OKADA *et al.* 2000), and the Y-specific primers for 4G7-T7 were used to score the male sex of progeny individuals (OKADA *et al.* 2000).

Screening and aligning Y chromosome-derived PAC clones:

PAC clones were screened from the *M. polymorpha* male genomic PAC library (OKADA *et al.* 2000) by PCR assay with pooled PAC DNA (GREEN and OLSON 1990; CHINAULT 1994) using primers for *rbm27* (see below) under the same conditions as described above. Plasmid DNA of the PAC clones and the *rbm27* were digested with *Bam*HI and electrophoresed in 0.7% agarose gel with 1 \times TAE buffer, and then the gel was stained by SYBR Gold (Molecular Probes, Eugene, OR) to confirm that the RDA-derived fragment was carried by the PAC clone and to align the PAC clones. The overlap of PAC clones was also confirmed by PCR amplification with each PAC DNA as template, using respective sets of primers for the SP6 and T7 ends of pMM23-104E4 (termed esM104E4, 5'-GGACTCTTCG CAGTCATGTATTC and 5'-CCTGGTATTACGAGAGAGCT

GAAC; etM104E4, 5'-TCCTTGCCGTGAGAGTTGATGTTC and 5'-AGAGAAGGATTGGGAGCCATTGATT). The PCR condition was described above.

The sequence data presented in this article have been submitted to the DDBJ/EMBL/GenBank Data Libraries under accession nos. AB069714 for *rbm27*, AB069720 for *rhm12*, AB069780 for *rhm13*, AB069718 for *rgm6*, AB069717 for *rgm29*, AB069716 for *rgm108*, AB069715 for *rbf62*, and AB069719 for *rhf73*, and the SP6- and T7-end sequences of the DNA fragment cloned into pMM23-104E4 [104E4SP6 (AB069721) and 104E4T7 (AB069722)].

RESULTS AND DISCUSSION

Isolation of DNA fragments specific to the male or female by RDA: Although initial male and female amplicons were visually indistinguishable from each other, DNA fragments unique to male or female plants were observed in the third-round products of *Bam*HI, the fourth-round products of *Hind*III, and the third-round products of *Bgl*II (Figure 1, A–C). These differences between male and female RDA products reflect the potential enrichment of DNA fragments characteristic of

TABLE 1
List of sex chromosome-specific RDA clones

Clone	Insert DNA size (bp)	Linkage ^a	Primer	Amplification size (bp)
<i>rbm27</i> (<i>Bam</i> HI)	1202	Y	Forward CCAAGTGC GGCGCAGAATCAAGT Reverse TTCATCGCCCGCTATCACCTTC	663
<i>rhm12</i> (<i>Hind</i> III)	1000	Y	Forward GAGAGTATTTGCGATGCGTCAC Reverse CAAGGGCTCGAATCCATTTCT	476
<i>rhm13</i> (<i>Hind</i> III)	1131	Y	Forward ACGTTGACGCATAAGGACATAA Reverse ATACCGGAAGGCATTTTCATC	310
<i>rgm6</i> (<i>Bgl</i> II)	899	Y	Forward TATCTCCGGGACATCTTTCTCTCT Reverse TCTCTATGAGGGGATTTTATTATT	211
<i>rgm29</i> (<i>Bgl</i> II)	221	Y	Forward CGCTGTCCAATCTTTCAAATAA Reverse ACAGCGGAGAACTGACTGACCC	197
<i>rgm108</i> (<i>Bgl</i> II)	658	Y	Forward GGAGACCTCTATCACCGCTGAAACT Reverse CACCTGCGGAGACTTATCTTACCTG	361
<i>rbf62</i> (<i>Bam</i> HI)	2444	X	Forward CAATTGGGAAGATTTGACACTTAGC Reverse TTCCCAATTGAGTACGAGTAGTCCT	454
<i>rhf73</i> (<i>Hind</i> III)	1075	X	Forward TGACGACGAAGATGTGGATGAC Reverse GAAACTTGGCCGTGTGACTGA	406

^a Y, Y linked; X, X linked.

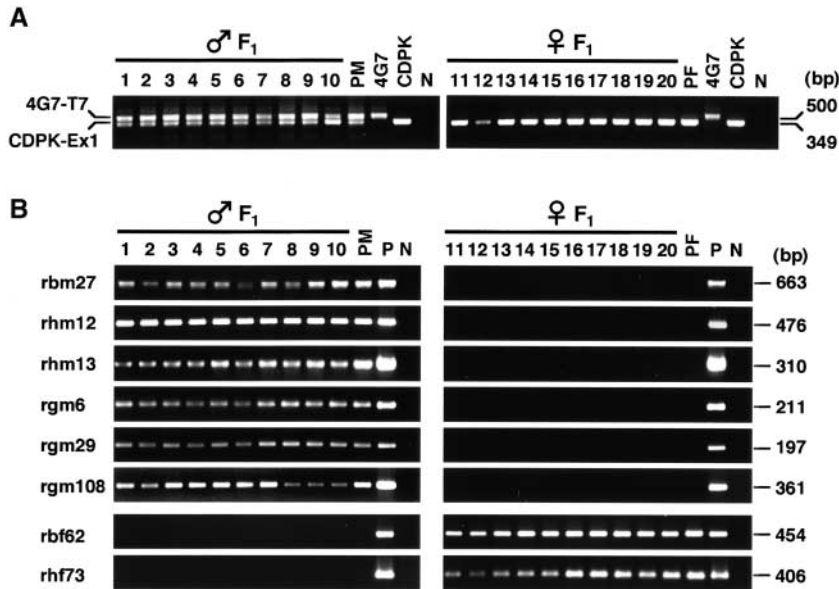


FIGURE 2.—Genetic linkage analysis of RDA-derived clones. (A) Sex of the F₁ progenies. Ten male individuals (left) and 10 females (right) were identified in the F₁. Lanes PM and PF, genomic DNA from parental male and female individuals; lanes 4G7 and CDPK, positive controls for respective primers; lane N, without template for a negative control. (B) Segregation of RDA-derived clones in the male and female F₁ progeny. Lanes 1–10, male F₁ progeny individuals; lanes 11–20, female F₁ progeny individuals. Lanes PM and PF, genomic DNA from parental male and female individuals. Lane P, the respective RDA-derived clones as template for a positive control. Lane N, without template for a negative control.

the male or the female genomes and therefore were investigated further.

The RDA products from the male and female genomic DNA were cloned into a cloning vector pBluescript II SK+ with the respective restriction enzymes and sequenced. For identification of male-specific RDA clones, 41 primer sets for the male RDA-derived clones (12 primer sets for *Bam*HI-RDA clones, 24 for *Hind*III-RDA clones, and 5 for *Bgl*II-RDA clones) were designed; for identification of female-specific RDA clones, 67 primer sets for the female RDA-derived clones (9 for *Bam*HI-

RDA clones, 53 for *Hind*III-RDA clones, and 5 for *Bgl*II-RDA clones) were designed. PCR test amplifications were performed on male and female genomic DNAs using each of these primer sets to determine whether the RDA-derived DNA fragments are indeed specific to either the parental male or female. Seven primer sets for male RDA-derived clones (2 for *Bam*HI-RDA clones, 2 for *Hind*III-RDA clones, and 3 for *Bgl*II-RDA clones) yielded PCR products in the parental male only and not in the parental female DNA (data not shown); 6 primer sets for female RDA-derived clones (2 for *Bam*HI-RDA

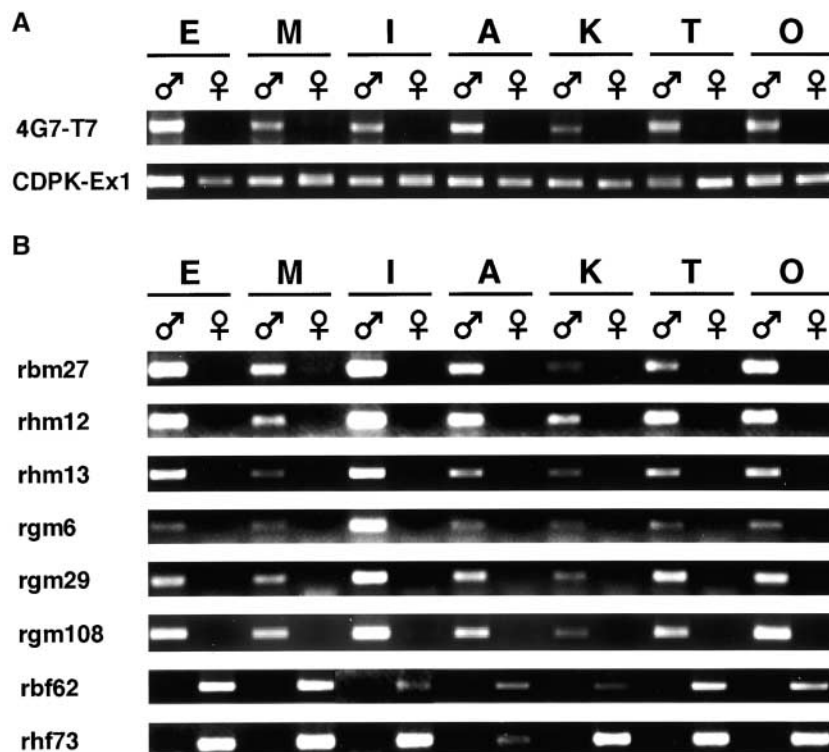


FIGURE 3.—Sex specificity of RDA-derived clones in the wild *M. polymorpha*. (A) Male specificity of the primers for 4G7-T7. (B) Sex specificity of RDA-derived clones. Lines E, M, I, A, K, T, and O were collected from different places in Japan. Lines E, M, and I were described by OKADA *et al.* (2000). The sex of these lines was checked by the presence of male or female sex organs.

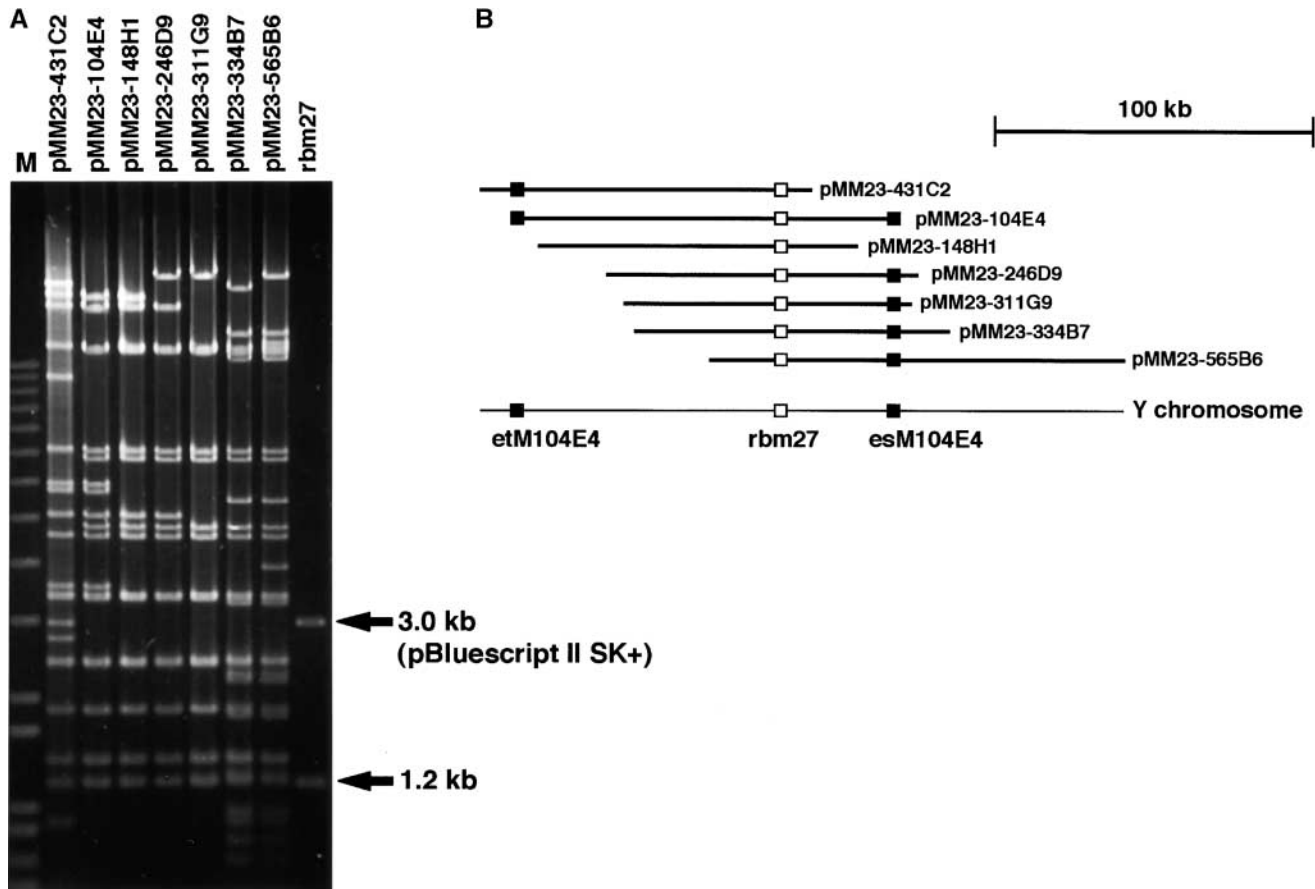


FIGURE 4.—PAC clones isolated by the primers for *rbm27* as a marker. (A) Agarose gel electrophoresis of PAC clones. Arrows indicate the RDA-derived DNA fragment of *rbm27* (1.2 kb) and a cloning vector pBluescriptII SK+ (3.0 kb), respectively. Lane M is the 1-kb plus DNA ladder (GIBCO BRL, Rockville, MD). (B) A PAC contig map of the region containing *rbm27* on the Y chromosome. PAC clones are indicated by labeled horizontal lines. Open squares indicate the amplification found with each PAC DNA as template by PCR using a set of primers for *rbm27*. Solid squares indicate the amplification found with each PAC DNA as template by PCR using respective sets of primers for the SP6- and T7-ends of pMM23-104E4 (termed esM104E4 and etM104E4, respectively). The exact position of *rbm27* in each PAC clone and the location of the contig on the Y chromosome are undefined.

clones, 3 for *Hind*III-RDA clones, and 1 for the *Bgl*II-RDA clone) amplified a product from the parental female, but not the parental male, DNA (data not shown).

Identification of RDA-derived DNA fragments specific to the sex chromosomes by genetic linkage analysis: Since the male and female plants used for RDA in this study were collected from wild populations, it was necessary to test whether the seven male-derived primer sets and six female-derived primer sets we confirmed are indeed specific to the male and female sex chromosomes, respectively. To do this, we performed cosegregation analysis using male and female F_1 progeny individuals. These were generated by crossing the male and female parents used for RDA isolation, and the sex of progeny plant was determined using PCR with the male-specific primer set 4G7-T7 (OKADA *et al.* 2000). We also tested six more pairs of wild *M. polymorpha* collected from different localities in Japan and found that only male individuals were positive (Figure 3A), indicating that 4G7-T7 can be used for determining the sex of *M.*

polymorpha individuals. A total of 10 male and 10 female individuals from the F_1 family were used for linkage analysis. The primer sets for six male RDA-derived clones, termed *rbm27*, *rhml2*, *rhml3*, *rgm6*, *rgm29*, and *rgm108* (Table 1), showed amplification only in the 10 male progeny, not in the females (Figure 2B). On the other hand, the primer sets for two female RDA-derived clones, termed *rbf62* and *rhf73* (Table 1), showed amplification only in the 10 females (Figure 2B). These results are statistically highly significant evidence for linkage. Using the genomic DNAs from wild *M. polymorpha* plants as templates, the above primer sets were further confirmed to be sex specific (Figure 3B).

Sequence analysis of the RDA-derived DNA fragments specific to the sex chromosomes: The nucleotide sequences of the six Y chromosome-specific DNA fragments (*rbm27*, *rhml2*, *rhml3*, *rgm6*, *rgm29*, and *rgm108*) showed no significant similarities to known sequences registered in the public databases as searched by BLAST (ALTSCHUL *et al.* 1997). Furthermore, none of these

Y chromosome-specific sequences display an extended reading frame, suggesting that they are not derived from protein-coding regions.

The nucleotide sequence of the X chromosome-specific DNA fragment rbf62 contains a 5S rRNA gene, indicating that this gene is present in the X chromosome as well as in the autosomes. SONE *et al.* (1999) reported the presence of the 5S rRNA gene in the 45S rDNA unit of *M. polymorpha*, and NAKAYAMA *et al.* (2001) showed that an rDNA cluster is found on the X chromosome, but not on the Y chromosome. The flanking regions of the 5S rRNA gene sequence in rbf62 are different from the rDNA unit previously reported (SONE *et al.* 1999), and it is not clear whether the rDNA unit was derived from autosomes or from the X chromosome. The linkage of rbf62 to the X chromosome implies that the flanking regions of the 5S rRNA gene sequence on the X chromosome have evolved differences from the autosomal units.

The nucleotide sequence of another X-linked sequence, rhf73, is found in two *M. polymorpha* expressed sequence tags (ESTs), F01I154 and F01Q066 (GenBank accession nos. C96067 and C96366, respectively), which had been isolated from immature female sex organs (NAGAI *et al.* 1999). Neither the rhf73 nor the two ESTs show similarity to sequences registered in the public databases.

The utility of RDA-derived DNA fragments as markers: To apply RDA-derived DNA fragments as markers to isolation of larger genomic DNA fragments, PAC clones from a male genomic library (OKADA *et al.* 2000) were screened by PCR using a set of primers for one of the Y chromosome-specific DNA fragments, rbm27. Seven PAC clones were isolated from the male genomic PAC library, which contains 22,000 clones. The cloning frequency of rbm27 (1 out of ~3000 clones) suggests that this sequence is present as a single genomic locus, since this library covers the male genome sevenfold (OKADA *et al.* 2000). A contig map of these PAC clones was constructed from their restriction profiles and the PCR amplification, using sets of primers for both end sequences of one of the PAC clones, pMM23-104E4. The map indicates that these PAC clones cover a single common overlapping region of the genome, thus further substantiating that rbm27 is derived from a unique Y chromosome locus (Figure 4, A and B).

We thank Dr. A. Brennicke, Ulm University, Germany, for his valuable suggestions and critical reading of the manuscript. This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology (no. 09306006) and by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

LITERATURE CITED

- AINSWORTH, C., J. PARKER and V. BUCHANAN-WOLLASTON, 1998 Sex determination in plants. *Curr. Top. Dev. Biol.* **38**: 167–223.
- ALTSCHUL, S. F., T. L. MADDEN, A. A. SCHÄFFER, J. ZHANG, Z. ZHANG *et al.*, 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**: 3389–3402.
- BISCHLER, H., 1986 *Marchantia polymorpha* L. S. LAT. karyotype analysis. *J. Hattori Bot. Lab.* **60**: 105–117.
- BUŽEK, J., H. KOUTNÍKOVÁ, A. HOUBEN, K. ŘÍHA, B. JANOUŠEK *et al.*, 1997 Isolation and characterization of X chromosome-derived DNA sequences from a dioecious plant *Melandrium album*. *Chromosome Res.* **5**: 57–65.
- CHINAULT, A. C., 1994 Screening large-insert libraries by PCR, pp. 5.5.1–10 in *Current Protocols in Human Genetics*, edited by N. C. DRACOPOLI, J. L. HAINES, B. R. KORF, D. T. MOIR, C. C. MORTON *et al.* John Wiley & Sons, New York.
- DELICHÈRE, C., J. VEUSKENS, M. HERNOULD, N. BARBACAR, A. MOURAS *et al.*, 1999 *SIY1*, the first active gene cloned from a plant Y chromosome, encodes a WD-repeat protein. *EMBO J.* **18**: 4169–4179.
- DONNISON, I. S., J. SIROKY, B. VYSKOT, H. SAEDLER and S. R. GRANT, 1996 Isolation of Y chromosome-specific sequences from *Silene latifolia* and mapping of male sex-determining genes using representational difference analysis. *Genetics* **144**: 1893–1901.
- GREEN, E. D., and M. V. OLSON, 1990 Systematic screening of yeast artificial-chromosome libraries by use of the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **87**: 1213–1217.
- GUTTMAN, D. S., and D. CHARLESWORTH, 1998 An X-linked gene with a degenerate Y-linked homologue in a dioecious plant. *Nature* **393**: 263–266.
- JUAREZ, C., and J. A. BANKS, 1998 Sex determination in plants. *Curr. Opin. Plant Biol.* **1**: 68–72.
- LISITSYN, N., N. LISITSYN and M. WIGLER, 1993 Cloning the differences between two complex genomes. *Science* **259**: 946–951.
- MATSUNAGA, S., S. KAWANO, T. MICHIMOTO, T. HIGASHIYAMA, S. NAKAO *et al.*, 1999 Semi-automatic laser beam microdissection of the Y chromosome and analysis of Y chromosome DNA in a dioecious plant, *Silene latifolia*. *Plant Cell Physiol.* **40**: 60–68.
- NAGAI, J., K. T. YAMATO, M. SAKAIDA, H. YODA, H. FUKUZAWA *et al.*, 1999 Expressed sequence tags from immature female sexual organ of a liverwort, *Marchantia polymorpha*. *DNA Res.* **6**: 1–11.
- NAKAYAMA, S., M. FUJISHITA, T. SONE and K. OHYAMA, 2001 Additional locus of rDNA sequence specific to the X chromosome of the liverwort, *Marchantia polymorpha*. *Chromosome Res.* **9**: 469–473.
- NISHIYAMA, R., H. MIZUNO, S. OKADA, T. YAMAGUCHI, M. TAKENAKA *et al.*, 1999 Two mRNA species encoding calcium-dependent protein kinases are differentially expressed in sexual organs of *Marchantia polymorpha* through alternative splicing. *Plant Cell Physiol.* **40**: 205–212.
- OKADA, S., M. FUJISAWA, T. SONE, S. NAKAYAMA, R. NISHIYAMA *et al.*, 2000 Construction of male and female PAC genomic libraries suitable for identification of Y-chromosome-specific clones from the liverwort, *Marchantia polymorpha*. *Plant J.* **24**: 421–428.
- OKADA, S., T. SONE, M. FUJISAWA, S. NAKAYAMA, M. TAKENAKA *et al.*, 2001 The Y chromosome in the liverwort *Marchantia polymorpha* has accumulated unique repeat sequences harboring a male-specific gene. *Proc. Natl. Acad. Sci. USA* **98**: 9454–9459.
- SCUTT, C. P., Y. KAMISUGI, F. SAKAI and P. M. GILMARTIN, 1997 Laser isolation of plant sex chromosomes: studies on the DNA composition of the X and Y sex chromosomes of *Silene latifolia*. *Genome* **40**: 705–715.
- SHIBATA, F., M. HIZUME and Y. KUROKI, 1999 Chromosome painting of Y chromosomes and isolation of a Y chromosome-specific repetitive sequence in the dioecious plant *Rumex acetosa*. *Chromosoma* **108**: 266–270.
- SONE, T., M. FUJISAWA, M. TAKENAKA, S. NAKAGAWA, S. YAMAOKA *et al.*, 1999 Bryophyte 5S rDNA was inserted into 45S rDNA repeat units after the divergence from higher land plants. *Plant Mol. Biol.* **41**: 679–685.
- STRAUS, D., and F. M. AUSUBEL, 1990 Genomic subtraction for cloning DNA corresponding to deletion mutations. *Proc. Natl. Acad. Sci. USA* **87**: 1889–1893.

Communicating editor: D. CHARLESWORTH